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RESEARCH ARTICLE

Reprogramming *Hansenula polymorpha* for penicillin production: expression of the *Penicillium chrysogenum* *pcl* gene

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β -lactam antibiotics; filamentous fungi; genetic
engineering; microbody; PTS1 receptor.

Introduction

Penicillin and its derivatives are the oldest known chemotherapeutic agents that are used against various bacteria. Penicillin belongs to the β -lactam family of antibiotics. The penicillin biosynthetic pathway in the filamentous fungus *Penicillium chrysogenum* has been well characterized both genetically and biochemically (Liras & Martin, 2006). In *P. chrysogenum*, this pathway is partly compartmentalized, and involves the action of the nonribosomal peptide synthetase δ -(L- α aminoadipyl)-L-cysteinyl-D-valine synthase and isopenicillin-N-synthase, which are present in the cytosol. These enzymes catalyze the first steps in β -lactam biosynthesis. In addition, the enzymes required in the final steps in penicillin biosynthesis, acyl-CoA:isopenicillin-N-acyltransferase (IAT) and phenylacetyl-CoA ligase (PCL), are located in microbodies (reviewed by van de Kamp *et al.*, 1999).

Microbodies (peroxisomes, glyoxysomes, glycosomes, Woronin bodies; in the remainder of the text, designated peroxisomes) are important organelles that are present in all eukaryotic cells. These organelles contain a protein-rich matrix consisting of enzymes involved in highly diverse metabolic pathways, such as plasmalogen biosynthesis in mammals, photorespiration in plants, glycolysis in trypano-

Abstract

We aim to introduce the penicillin biosynthetic pathway into the methylotrophic yeast *Hansenula polymorpha*. To allow simultaneous expression of the multiple genes of the penicillin biosynthetic pathway, additional markers were required. To this end, we constructed a novel host–vector system based on methionine auxotrophy and the *H. polymorpha* *MET6* gene, which encodes a putative cystathionine β -lyase. With this new host–vector system, the *Penicillium chrysogenum* *pcl* gene, encoding peroxisomal phenylacetyl-CoA ligase (PCL), was expressed in *H. polymorpha*. PCL has a potential C-terminal peroxisomal targeting signal type 1 (PTS1). Our data demonstrate that a green fluorescent protein–PCL fusion protein has a dual location in the heterologous host in the cytosol and in peroxisomes. Mutation of the PTS1 of PCL (SKI-COOH) to SKL-COOH restored sorting of the fusion protein to peroxisomes only. Additionally, we demonstrate that peroxisomal PCL–SKL produced in *H. polymorpha* displays normal enzymatic activities.

somes, the primary metabolism of various unusual carbon sources, as well as penicillin biosynthesis in certain filamentous fungi (van den Bosch *et al.*, 1992).

We aim to introduce the penicillin biosynthetic pathway in the methylotrophic yeast *Hansenula polymorpha*. Yeast species have the advantage of being versatile, and easy to handle and cultivate. The use of a yeast model system provides information on the actual requirements of the penicillin biosynthetic pathway. Additionally, it enables easy manipulation of the penicillin biosynthetic pathway for the production of novel antibiotics. *Hansenula polymorpha* has been successfully developed as a host for the production of foreign proteins (Hollenberg & Gellissen, 1997). *Hansenula polymorpha* has the added advantage that the number and volume fraction of peroxisomes can be readily regulated, allowing better control over peroxisomal enzymes and their function (van der Klei *et al.*, 1991).

Proper sorting of the peroxisome-borne enzymes IAT and PCL to the correct destination in the yeast host is of crucial importance in this metabolic reprogramming scheme. This has already been achieved for *P. chrysogenum* IAT. When produced in *H. polymorpha*, IAT is correctly sorted to peroxisomes and functionally active (Lutz *et al.*, 2005). In the current work, we introduced the *P. chrysogenum* *pcl* gene

into *H. polymorpha* using a novel host–vector system, and determined the subcellular location and activity of the heterologously produced protein.

Materials and methods

Microorganisms and growth conditions

The strains used in this study are listed in Table 1. All *H. polymorpha* strains are derivatives of NCYC495 (Gleeson & Sudbery, 1988), and were grown at 37 °C in either (1) rich complex media (YPD) containing 1% yeast extract, 1% peptone and 1% glucose, (2) selective media containing 0.67% yeast nitrogen base without amino acids (DIFCO) supplemented with 0.5% glucose (YND) or 0.5% methanol (YNM), or (3) mineral medium (MM) as described by Van Dijken *et al.* (1976), supplemented with 0.25% ammonium sulfate, using 0.5% glucose or 0.5% methanol as carbon source. For growth on plates, 2% granulated agar was added to the media. Whenever necessary, media were supplemented with 30 µg mL⁻¹ leucine, 30 µg mL⁻¹ uracil, 20 µg mL⁻¹ adenine, and 20 µg mL⁻¹ methionine. For biochemical analysis, selected strains were precultured for at least three rounds in MM containing glucose, and subsequently shifted to MM containing methanol to induce expression of genes under the control of the alcohol oxidase (AO) promoter. For

cloning purposes, *Escherichia coli* DH5α (Gibco-BRL, Gaithersburg, MD) was used, and grown at 37 °C in Luria–Bertani medium (1% Bacto tryptone, 0.5% yeast extract, 0.5% NaCl), supplemented with 100 µg mL⁻¹ ampicillin or 25 µg mL⁻¹ kanamycin when required.

Construction of *H. polymorpha* NCYC495 *ade11.1 leu1.1 met6 ura3*

To obtain a fourfold auxotrophic *H. polymorpha* strain, we crossed NCYC495 *ade11.1 leu1.1 ura3* (Haan *et al.*, 2002) with NCYC495 *ade11.1 leu1.1 met6*, using the procedure described by Gleeson & Sudbery (1988). This resulted in the isolation of strain NCYC495 *ade11.1 leu1.1 met6 ura3*.

Miscellaneous DNA techniques

The plasmids and primers used in this study are listed in Tables 1 and 2, respectively. All DNA manipulations were carried out according to standard methods (Sambrook *et al.*, 1989). *Hansenula polymorpha* cells were transformed by electroporation (Faber *et al.*, 1994). Chromosomal DNA was extracted from YPD-grown *H. polymorpha* cells as described by Sherman *et al.* (1986), but included an additional protein precipitation step using 5 M sodium chloride prior to DNA precipitation. DNA-modifying enzymes were used as recommended by the supplier (Roche, Almere, the

Table 1. Strains and plasmids used in this study

Strain/plasmids	Genotype/description	Reference
<i>E. coli</i>		
DH5α	<i>supE44 ΔlacU169(φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook <i>et al.</i> (1989)
<i>H. polymorpha</i>		
NCYC495 <i>ade11.1 leu1.1 ura3</i>	Adenine, leucine and uracil auxotrophic strain	Haan <i>et al.</i> (2002)
NCYC495 <i>ade11.1 leu1.1 met6</i>	Adenine, leucine and methionine auxotrophic strain	Laboratory collection
NCYC495 <i>ade11.1 leu1.1 ura3 met6</i>	Segregant of cross between NCYC495 <i>ade11.1 leu1.1 ura3</i> and NCYC495 <i>ade11.1 leu1.1 met6</i>	This study
<i>P. chrysogenum</i>		
DS17690	High penicillin-producing strain	DSM anti-infectives, Delft, the Netherlands
Plasmids		
pYT3-MET6	Plasmid complementing the methionine auxotrophy of <i>H. polymorpha ade11.1 leu1.1 met6</i> ; amp ^R	This study
pBluescript II SK ⁺	<i>E. coli</i> cloning vector; amp ^R	Stratagene Inc., San Diego, CA
pHIPX4	<i>H. polymorpha</i> integrating plasmid; <i>Sc-LEU2</i> ; kan ^R	Gietl <i>et al.</i> (1994)
pHIPX4-HNBESX	Derivative of pHIPX4 with alternative polylinker; <i>Sc-LEU2</i> ; kan ^R	Laboratory collection
pSKMET6	pBluescript II SK ⁺ containing the <i>H. polymorpha MET6</i> gene; amp ^R	This study
pHIPM4	<i>H. polymorpha</i> integrating plasmid; <i>Hp-MET6</i> ; kan ^R	This study
pHIPM4-PCL	pHIPM4 containing <i>P. chrysogenum pcl</i> ; <i>Hp-MET6</i> ; kan ^R	This study
pHIPM4-GFP.PCL	pHIPM4 containing fusion gene between <i>GFP</i> and <i>P. chrysogenum pcl</i> ; <i>Hp-MET6</i> ; kan ^R	This study
pHIPM4-PCL.SK1	pHIPM4 containing <i>P. chrysogenum pcl.SK1</i> ; <i>Hp-MET6</i> ; kan ^R	This study
pHIPM4-GFP.PCL.SK1	pHIPM4 containing fusion gene between <i>GFP</i> and <i>P. chrysogenum pcl.SK1</i> ; <i>Hp-MET6</i> ; kan ^R	This study

Table 2. Oligonucleotide primers used in this study

Primer name	Primer sequence
MET6F	5'-AGAGGATCCGCGACCGGTTGGTTATC-3'
MET6R	5'-GCCTATAAAGGGGCGCTTTG-3'
PCL-1F	5'-CTAGGATCCTAGGTTTTTACCTCCAAAGGAG-3'
PCL-3R	5'-AGTCTCGAGTTAGATCTTGCTACCAGCCTTTC-3'
PCL-4R	5'-GGAAGATCTGTCGACTTACAGCTTGCTACCAGCCTTTC-3'
GFP-F	5'-CCCAAGCTTGGGATGGTGAGCAAGGGCGAGGAGC-3'
GFP-R	5'-ATGCCATGGCCTTGACAGCTCGTCCATG-3'

Netherlands). Pwo polymerase was used for preparative PCR. Southern blot analysis was performed with the ECL direct nucleic acid labeling and detection system (Amersham Corp., Arlington Heights, IL). Oligonucleotides were synthesized by Life Technologies (Breda, the Netherlands). DNA sequencing reactions were performed at BaseClear (Leiden, the Netherlands). For DNA sequence analysis, the CLONE MANAGER 5 program (Scientific and Educational Software, Durham) was used. BLAST algorithms (Altschul *et al.*, 1990) were used to screen databases at the National Center for Biotechnology Information (Bethesda, MD). The CLUSTAL_X program was used to align protein sequences (Thompson *et al.*, 1997), and the GENEDOC program (available at <http://www.psc.edu/biomed/genedoc>) was used to display the aligned sequences.

Construction of plasmids

The novel *H. polymorpha* integration vector pHIPM4 was constructed as follows. First, a 2.0-kb DNA fragment comprising the *H. polymorpha* MET6 gene, including its regulatory sequences, was isolated by PCR with primers MET6F and MET6R (Table 2), using pYT3-MET6 as template, and cloned into SmaI-digested pBluescript II SK+. From the resulting plasmid, designated pSKMET6, a 2.0-kb BamHI fragment containing *HpMET6* was isolated and cloned into BglII-digested pHIPX4-HNBESX [a derivative of pHIPX4 (Gietl *et al.*, 1994)], thereby replacing the *ScLEU2* marker in this plasmid. The resulting plasmid was designated pHIPM4 (Fig. 2).

For the construction of plasmid pHIPM4-PCL, a BamHI site was introduced upstream of the *P. chrysogenum* *pcl* gene by PCR with primers PCL-1F and PCL-3R, using DNA from a *P. chrysogenum* cDNA library (Kiel *et al.*, 2000) as template. The resulting 1.7-kb DNA fragment was digested with BamHI, and cloned between the BamHI and SmaI sites of pHIPM4, resulting in plasmid pHIPM4-PCL.

To construct pHIPM4-GFP.PCL, we amplified the *GFP* gene by PCR using primers GFP-F and GFP-R. The resulting 717-bp product was then digested with HindIII and NcoI, and cloned between the HindIII and NcoI sites of plasmid pHIPM4-PCL. The resulting plasmid, designated pHIPM4-GFP.PCL, contains an in-frame *GFP-pcl* fusion gene.

To enable optimization of the targeting of PCL to peroxisomes, we mutagenized the PTS1 signal of PCL (SKI-COOH) into SKL-COOH. To this end, the *P. chrysogenum* *pcl* gene was amplified by PCR with primers PCL-1F and PCL-4R, using pHIPM4-PCL as template. The resulting 1.7-kb DNA fragment, containing the *pcl*.SKL coding sequence, was inserted between the BamHI and SmaI sites of pHIPM4, resulting in plasmid pHIPM4-PCL.SKL. In addition, the same fragment was inserted between the BamHI and SmaI sites of pHIPM4-GFP.PCL, resulting in plasmid pHIPM4-GFP.PCL.SKL.

Integration of pHIPM4-derived plasmids into the *MET6* locus of the *H. polymorpha* genome was achieved by transforming EcoRI-linearized plasmid DNA. Correct integration and copy number determination was analyzed by Southern blotting (data not shown).

Biochemical methods

Crude extracts of *H. polymorpha* and *P. chrysogenum* cells were prepared with glass beads basically as described by Waterham *et al.* (1994), using TANG buffer (50 mM Tris-HCl, pH 7.5, 0.02% sodium azide, 200 mM NaCl, 10% glycerol) instead of phosphate buffer. For Western blots, extracts of *H. polymorpha* and *P. chrysogenum* cells were prepared using the trichloroacetic acid (TCA) method (Baerends *et al.*, 2000). Protein concentrations were determined using the Bio-Rad Protein Assay system and bovine serum albumin as standard. Sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western blotting were performed using established procedures. Western blots were decorated with polyclonal antibodies raised in rabbit against a maltose binding protein (MBP)-PCL fusion protein produced in *E. coli* (a gift of M. Koetsier, Biochemical Laboratory, GBB, University of Groningen, the Netherlands).

Determination of PCL enzymatic activity

PCL activity was determined in crude cell extracts by measuring the rate of conversion of cinnamic acid to cinnamoyl-CoA at 30 °C, following the procedure of M. Koetsier *et al.* (unpublished results).

Morphologic analysis

Fluorescence microscopy studies were performed using a Zeiss Axioskop microscope (Carl Zeiss, Göttingen, Germany).

Results

Cloning of *H. polymorpha* MET6

Introduction of the *P. chrysogenum* penicillin biosynthetic pathway into *H. polymorpha* involves expression of the multiple *P. chrysogenum* genes of this pathway (Brakhage,

1998). To allow the integration of multiple expression cassettes into *H. polymorpha*, a new vector utilizing the *MET6* gene as a novel auxotrophic marker was first constructed. In order to isolate the *H. polymorpha* *MET6* gene, we transformed strain NCYC495 *ade11.1 leu1.1 met6* with an *H. polymorpha* genomic library in pYT3 (Tan *et al.*, 1995). From a methionine prototrophic transformant, a plasmid with an insert of 6.1 kb was rescued in *E. coli*. Subsequent retransformation of NCYC495 *ade11.1 leu1.1 met6* with the isolated plasmid confirmed its complementing ability. Initial DNA sequence analysis of the insert revealed the presence of an ORF with high similarity to the *Saccharomyces cerevisiae* *STR3* gene product, which is involved in methionine biosynthesis. Subcloning confirmed that this gene can complement the methionine auxotrophy of *H. polymorpha* NCYC495 *ade11.1 leu1.1 met6*. Hence, we designated this gene *H. polymorpha* *MET6*. The sequence of *H. polymorpha* *MET6* was submitted to Genbank and was assigned the accession number EF035078.

Hansenula polymorpha *MET6* encodes a protein of 384 amino acids with a predicted molecular mass of 42 kDa. BLAST analysis showed highest similarity to cystathionine β -lyase (CBL), an enzyme involved in the conversion of cystathionine into homocysteine (Hansen & Johannesen, 2000). The *H. polymorpha* CBL protein is 43% identical to its *S. cerevisiae* ortholog. An alignment of selected CBLs from a number of organisms is shown in Fig. 1. The alignment demonstrates that the amino acids involved in catalytic activity and cofactor (pyridoxal 5'-phosphate) binding are well conserved in *H. polymorpha* CBL. Previously, it has been demonstrated that *S. cerevisiae* CBL localizes to peroxisomes (Schafer *et al.*, 2001). *Saccharomyces cerevisiae* CBL contains a C-terminal SKL-COOH, a typical type 1 peroxisomal targeting signal (PTS1) (Purdue & Lazarow, 2001). Further analysis showed that orthologs from yeast species that are evolutionarily close to baker's yeast, i.e. *Candida glabrata*, *Kluyveromyces lactis*, *Ashbya gossypii*, *C. albicans*, and *Debaromyces hansenii*, also contain a putative PTS1 (data not shown). This suggests that, in these organisms, methionine biosynthesis is partly compartmentalized. Remarkably, the *H. polymorpha* CBL lacks a PTS1 (Fig. 1). In this respect, this protein more resembles CBL from *Yarrowia lipolytica* and filamentous fungi.

Construction of an *H. polymorpha* strain producing *P. chrysogenum* PCL

We utilized the *H. polymorpha* *MET6* gene with its endogenous promoter and terminator regions as auxotrophic marker in the construction of a novel *H. polymorpha* integration vector, designated pHIPM4 (Fig. 2). This vector allows high-level expression of genes by the strong, inducible AO promoter (P_{AOX}), and also contains the amine

oxidase terminator (T_{AMO}). Integration of pHIPM4 into the *H. polymorpha* genome can be facilitated by linearization in either the P_{AOX} or *MET6* regions.

To fully explore the potential of pHIPM4, a fourfold auxotrophic strain, NCYC495 *ade11.1 leu1.1 ura3 met6*, was constructed. To demonstrate the use of this new host-vector system in metabolic engineering of *H. polymorpha*, we inserted the gene encoding *P. chrysogenum* PCL downstream from the P_{AOX} in pHIPM4. The resulting plasmid, pHIPM4-PCL, was then integrated at the *MET6* locus of strain NCYC495 *ade11.1 leu1.1 ura3 met6*. Initially, a strain containing a single copy of the PCL expression cassette, designated PCL^{1c}, was analyzed further. Western blots, using crude extracts prepared from methanol-grown cells, decorated with α -PCL antibodies, demonstrated (Fig. 3, lanes 1–3) that strain PCL^{1c} produces high levels of a protein identical in size to *P. chrysogenum* PCL that was absent in wild-type controls. From this, we conclude that the novel host-vector system enables efficient production of heterologous proteins.

Localization of *P. chrysogenum* PCL in *H. polymorpha*

In *P. chrysogenum*, PCL localizes to peroxisomes (W.H. Meijer *et al.*, unpublished results), probably via its PTS1 signal, SKI-COOH. In order to determine the subcellular location of PCL in *H. polymorpha*, we constructed strain GFP.PCL expressing a P_{AOX} -driven GFP-*pcl* fusion gene. Methanol-grown cells of this strain were analyzed by fluorescence microscopy. The data (Fig. 4a and b) indicate that in *H. polymorpha*, GFP-PCL has a dual localization. GFP fluorescence was observed in peroxisomes as a characteristic rim that surrounds the AO crystal (Veenhuis *et al.*, 2000), but was also detected in the cytosol. To sort PCL completely to peroxisomes of *H. polymorpha*, the native PTS1 signal of PCL (SKI-COOH) was changed to SKL-COOH, which is a more efficient PTS1 signal (Reumann, 2004). In the resulting strain, GFP.PCL^{SKL}, GFP fluorescence was confined to peroxisomes (Fig. 4c and d).

PCL^{SKL} produced in *H. polymorpha* is active

For enzymatic activity determinations, the *pcl*^{SKL} gene was inserted into plasmid pHIPM4 and integrated with varying copy numbers into *H. polymorpha* NCYC495 *ade11.1 leu1.1 ura3 met6*. Strains containing one, two and multiple (more than three) copies of the expression cassette were selected. Western blot analysis demonstrated that these strains produced enhanced levels of PCL^{SKL} protein, corresponding to the number of expression cassettes integrated (Fig. 3, lanes 4–6).

PCL is involved in the activation of aromatic monocarboxylic acids by covalently adding CoA to the substrate (Ward & KE, 2005). We determined the enzymatic activity of PCL^{SKL}

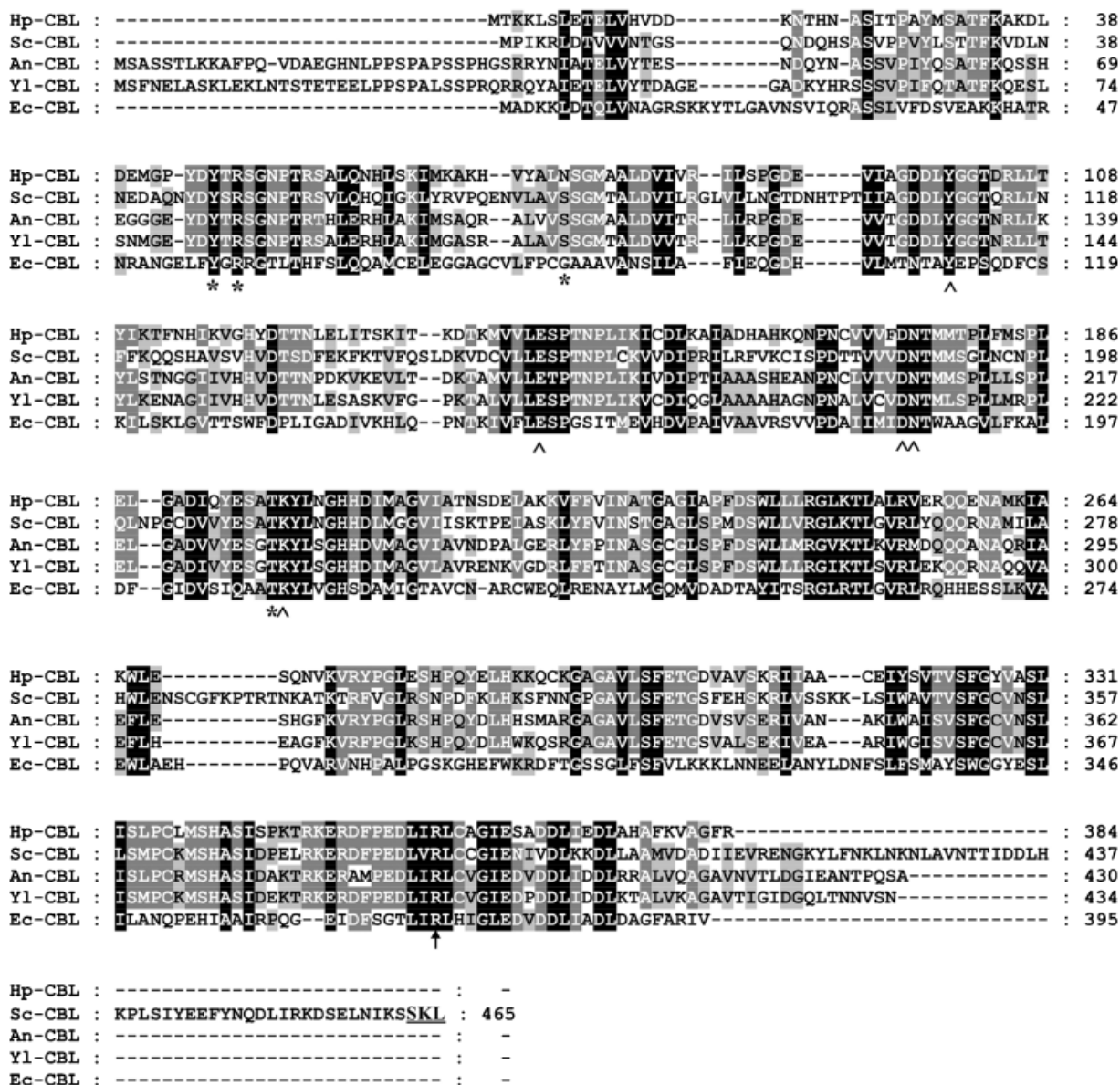


Fig. 1. Sequence alignment of the putative CBL encoded by *Hansenula polymorpha* MET6 (Hp) with those from *Saccharomyces cerevisiae* (Sc: Genbank accession number NP011331), *Yarrowia lipolytica* (Yl: Genbank accession number CAG80434), *Escherichia coli* (Ec: Genbank accession number NP417481), and *Aspergillus nidulans* (An). The correct *A. nidulans* CBL protein sequence was deduced from accession number AACD01000117 with two introns removed as described by Sienko & Paszewski (1999). Gaps were introduced to maximize the similarity. Identical and conserved residues are shaded. The arrow points to arginine 372 in *E. coli* CBL, which is the general α -carboxylate binding group of all CBL proteins. ^, points at amino acids 111, 154, 185, 186 and 210 of *E. coli* CBL, which are involved in catalysis. The asterisks indicate amino acids 56, 58, 86 and 209 of *E. coli* CBL, which are involved in cofactor binding. The C-terminal PTS1 signal present in *S. cerevisiae* CBL is underlined.

in methanol-grown cells of strains PCL^{SKL,1c}, PCL^{SKL,2c} and PCL^{SKL,mc} with cinnamic acid as substrate, using *H. polymorpha* host cells and *P. chrysogenum* DS17690 cells as controls. The results (Table 3) indicated that crude extracts of the recombinant *H. polymorpha* strains displayed PCL enzymatic activity levels that increased with increasing copy numbers of the *pcSKL* expression cassette. PCL enzymatic activity was absent in the control host strain. The PCL activities detected in

the *H. polymorpha* recombinant strains were enhanced relative to the activities in *P. chrysogenum* cells.

Discussion

Penicillin is normally produced by the filamentous fungus *P. chrysogenum*. Introduction of the penicillin biosynthetic pathway into a yeast species is beneficial for overcoming the

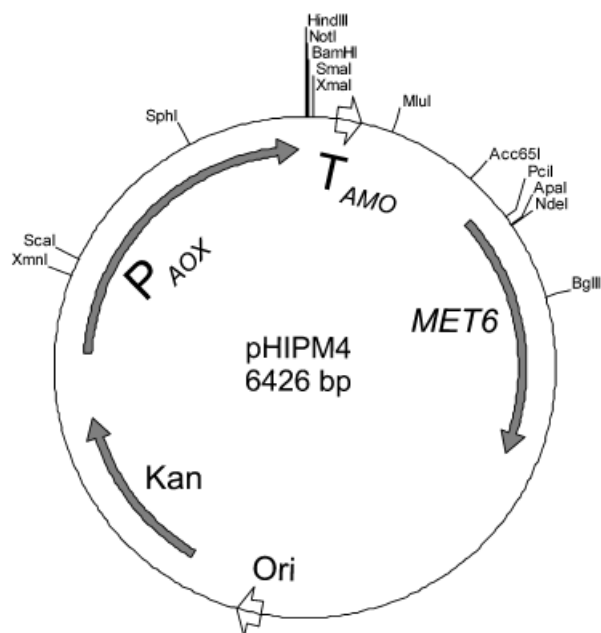


Fig. 2. Physical map of the *Hansenula polymorpha* integrating plasmid pHIPM4. Indicated are: P_{AOX} , *H. polymorpha* AO promoter; T_{AMO} , *H. polymorpha* amine oxidase terminator; *MET6*, *H. polymorpha* *MET6* gene with its endogenous promoter and terminator regions; Kan, kanamycin resistance gene; Ori, *Escherichia coli* origin of replication.

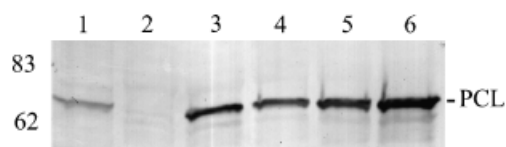


Fig. 3. Western blot analysis of strains producing PCL. *Hansenula polymorpha* cells were pregrown on glucose-containing medium, and subsequently cultivated for 16 h on methanol-containing medium to induce the *AOX* promoter. Additionally, *Penicillium chrysogenum* DS17690 cells were grown on penicillin production medium for 40 h to obtain optimal penicillin production. Cells were TCA-precipitated and prepared for Western blot analysis. Equal amounts of protein (corresponding to 20 µg) were loaded per lane. The blot was then decorated with polyclonal α -MBP-PCL antibodies. Sizes of marker proteins are indicated on the left (kDa). Lane 1, *P. chrysogenum* DS17690; lane 2, *H. polymorpha* host strain (NCYC495 *ade11.1 leu1.1 ura3 met6*); lane 3, *H. polymorpha* strain PCL^{1c} ; lane 4, *H. polymorpha* strain $PCL^{SKL, 1c}$; lane 5, *H. polymorpha* strain $PCL^{SKL, 2c}$; lane 6, *H. polymorpha* strain $PCL^{SKL, mc}$.

difficulties of using *P. chrysogenum* in bulk fermentations, avoiding strain instabilities, reducing intracellular proteases, etc. The methylotrophic yeast *H. polymorpha* has been successfully developed as a host for the production of foreign proteins (Gellissen & Hollenberg, 1997). High-level protein production is facilitated by the availability of strong, inducible promoters (P_{AOX} , P_{FMD}) (van Dijk *et al.*, 2000;

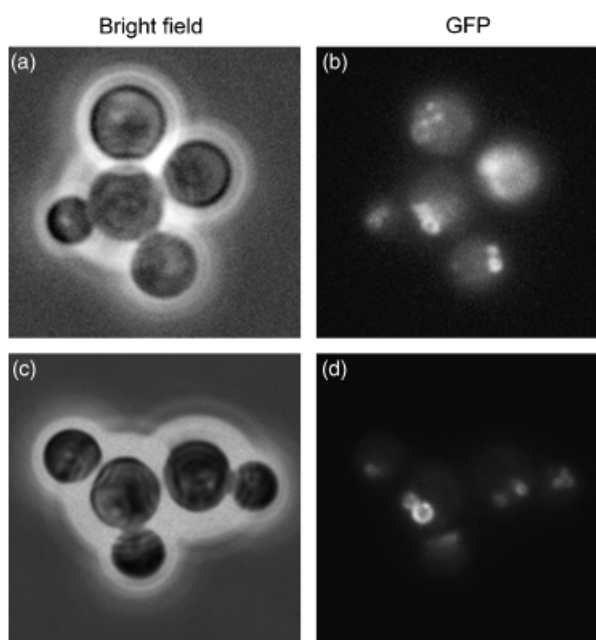


Fig. 4. Localization of GFP-PCL and GFP-PCL^{SKL} in *Hansenula polymorpha*. Fluorescence microscopy images of *H. polymorpha* cells producing GFP-PCL (a, b) and GFP-PCL^{SKL} (c, d) grown on methanol medium. (a, c) Bright field images. (b, d) Fluorescence images. In *H. polymorpha* cells producing GFP-PCL, fluorescence is observed in both peroxisomes and cytosol. In *H. polymorpha* cells producing GFP-PCL^{SKL}, fluorescence is present only in peroxisomes.

Table 3. PCL enzyme activity measurements

Strain	U mg ⁻¹
<i>H. polymorpha</i> NCYC495 <i>ade11.1 leu1.1 ura3 met6</i>	0.00
<i>H. polymorpha</i> $PCL^{SKL, 1c}$	0.96
<i>H. polymorpha</i> $PCL^{SKL, 2c}$	1.96
<i>H. polymorpha</i> $PCL^{SKL, mc}$	5.44
<i>P. chrysogenum</i> DS17690	0.05

1 U = 1 mM min⁻¹.

Gellissen & Veenhuis, 2001). We aimed to utilize the favorable properties of *H. polymorpha*, via metabolic reprogramming, for synthesis of β -lactam antibiotics.

Penicillin biosynthesis requires the activity of multiple enzymes that are not available in *H. polymorpha*. To enable the use of multiple expression cassettes in this yeast, we first constructed a new host-vector system that consisted of an *H. polymorpha* strain carrying four auxotrophic markers and the novel integration vector pHIPM4, which contains the *H. polymorpha* *MET6* gene. This novel host-vector system provides an additional tool for efficient metabolic engineering of *H. polymorpha*. Its suitability was demonstrated by the efficient production of *P. chrysogenum* PCL. Our data indicate that the new vector can integrate in single and multiple copies into the *H. polymorpha* genome.

Southern blot analysis has shown that among 32 randomly picked transformants, in 22 cases the expression cassette had correctly integrated into the *MET6* locus. Of these, 64% contained one copy, 18% two copies and 18% multiple copies (three or more) of the expression cassette (data not shown). Thus, integration of varying copies of pHIPM4-derivatives allows efficient regulation of the expression level of a heterologous gene, as demonstrated by our expression data for *pcl*-SKL (Table 3 and Fig. 3).

In *P. chrysogenum*, the last steps in penicillin production, side chain activation and attachment of the activated side chain to the β -lactam backbone, are performed by the peroxisomal enzymes PCL and IAT, respectively (Muller *et al.*, 1992; Lamas-Maceiras *et al.*, 2006). Both proteins contain a PTS1 (PCL, SKI-COOH; IAT, ARL-COOH), indicating that sorting to *P. chrysogenum* peroxisomes requires the PTS1 receptor, Pex5p (Holroyd & Erdmann, 2001). Previously, we have demonstrated that IAT produced in *H. polymorpha* completely localizes to peroxisomes (Lutz *et al.*, 2005), implying efficient recognition of its PTS1 by *H. polymorpha* Pex5p. In contrast, our localization studies of GFP-PCL provide evidence that this protein is not efficiently sorted to peroxisomes of *H. polymorpha*. In *P. chrysogenum*, PCL completely localizes to peroxisomes (W.H. Meijer *et al.*, unpublished results). This suggests that *P. chrysogenum* Pex5p binds the PTS1 of PCL much more effectively than its *H. polymorpha* ortholog. Such differences in recognition of PTS1 sequences by different Pex5 proteins have been observed before (van der Klei *et al.*, 1995; Neuberger *et al.*, 2003). Alternatively, endogenously produced PCL may contain (an) additional binding site(s) for *P. chrysogenum* Pex5p that enables its efficient sorting to *P. chrysogenum* peroxisomes, and that is not recognized by *H. polymorpha* Pex5p. An analogous situation was observed in an *H. polymorpha* *pex5* strain producing *P. chrysogenum* Pex5p (Kiel *et al.*, 2004). In this study, *P. chrysogenum* Pex5p was unable to sort endogenous AO into peroxisomes, whereas other PTS1 proteins (including GFP-SKL) were efficiently imported. Presumably, *P. chrysogenum* Pex5p does not recognize the highly efficient, alternative PTS that resides in the AO molecule (Gunkel *et al.*, 2004; Kiel *et al.*, 2004). Thus, both PTS1 receptors have certain unique features. Nevertheless, efficient sorting of PCL to peroxisomes of *H. polymorpha* could be established when the PTS1 signal of the protein was changed to the canonical SKL-COOH. This confirms that the low efficiency of targeting of PCL in *H. polymorpha* was not caused by significant structural changes in the C-terminus of the protein that may have precluded binding of *H. polymorpha* Pex5p.

Our data show that *H. polymorpha* strains containing varying copies of the *pcl*^{SKL} expression cassette may produce significantly higher levels of enzymatic activity than observed in *P. chrysogenum*. Previously, it was shown that

overproduction of PCL in *P. chrysogenum* significantly increased penicillin production (Lamas-Maceiras *et al.*, 2006). This suggests that the endogenous levels of PCL in *P. chrysogenum* may not be optimal for efficient penicillin production. In *H. polymorpha*, such an optimization of PCL-SKL levels can easily be obtained, because multiple expression systems are available.

In conclusion, our data demonstrate that, in addition to *P. chrysogenum* IAT, PCL-SKL can also be produced in *H. polymorpha* in a functionally active form, and is properly targeted to peroxisomes. This provides new possibilities for the metabolic reprogramming of *H. polymorpha* to produce penicillin.

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